44. (new) A method of propagating adenovirus *in vitro*, the method comprising:

a replication competent adenovirus vector for selective cytolysis of a target cell, comprising an E2F-1 transcriptional regulatory element (TRE) operably linked to an adenovirus gene essential for replication selected from the group consisting of E1A, E1B and E4 wherein said cell is maintained under cell cycling conditions *in vitro*, thereby expressing said adenovirus gene essential for replication;

wherein said adenovirus is propagated.

45. (new) The method of Claim 44, wherein said propagating of said adenovirus is cytotoxic to said cell.

46. (new) The method of Claim 44, wherein said cell is a tumor cell.

REMARKS

In view of the above amendments and the following remarks, the Examiner is respectfully to allow claims 1, 8, 14-16, 21, 24-26, and 32-46, the currently pending claims.

Newly added claims 35-46 are supported in the specification on page 22, lines 3-16, which disclose the use of a cell-status specific TRE, where the TRE is associated with the cell cycle gene E2F-1. The 5' region of the human E2F-1 gene is provided by Applicants as SEQ ID NO:2, and is discussed in the cited reference, Johnson *et al.* (1994) *Genes Dev.* 8:1514-1525 (page 22, lines 5-6). Support for the language "a nucleotide sequence having at least 80% sequence identity"; may be found on page 24, lines 13-14; and the language "hybridizes under stringent conditions" may be found on page 24, lines 21-22. Claims 41-46 find support in previously filed claims 24, 25, 26 and 32, 33, 34, respectively.

One of skill in the art can practice the claimed invention without undue experimentation. The claimed adenovirus vectors, comprising an adenovirus gene essential for regulation under the transcriptional control of an E2F-1 transcriptional regulatory element are expected to provide preferential adenovirus replication under conditions where the Rb genes are inactivated, as discussed in the specification on page 22, lines 7-10. The attached article, Parr *et al.* (1997) Nat. Medicine 3:1145-1149, demonstrates an E2F-1-responsive adenoviral vector provides for tumor-selective expression of a transgene under the regulatory control of the E2F-1 TRE. De-repression of the E2F-1 promoter occurs *in vivo* in cancer cells.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached is captioned **"VERSION WITH MARKINGS TO SHOW CHANGES MADE."**
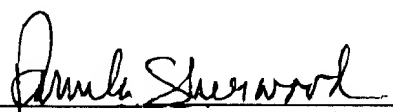
CONCLUSION

Applicants submit that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a Telephone Conference would expedite the prosecution of this application, she is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any other fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number CELL-014.

Respectfully submitted,

Date: March 4, 2002   By: _____
                                              Pamela J. Sherwood, Ph.D.
                                              Registration No. 36,677

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APPENDIX

VERSION WITH MARKINGS TO SHOW CHANGES MADE

35. (new) A replication-competent adenovirus vector for selective cytolysis of a target cell, comprising:

an E2F-1 transcriptional regulatory element (TRE) operably linked to an adenovirus gene essential for replication selected from the group consisting of E1A, E1B and E4.

36. (new) The adenovirus vector of claim 35, wherein the E2F-1 TRE is human.

37. (new) The adenovirus vector of Claim 36, wherein said E2F-1 TRE comprises the nucleotide sequence set forth in SEQ ID NO:2.

38. (new) The adenovirus vector of Claim 35, wherein said E2F-1 TRE comprises a nucleotide sequence having at least 80% sequence identity with the sequence set forth in SEQ ID NO:2.

39. (new) The adenovirus vector of Claim 35, wherein said E2F-1 TRE comprises a nucleotide sequence that hybridizes under stringent conditions with the sequence set forth in SEQ ID NO:2.

40. (new) The adenovirus vector of Claim 35, wherein said adenovirus gene essential for replication is operably linked to a composite regulatory element comprising said HRE and a cell-type specific transcriptional regulatory element (TRE).

41. (new) The adenovirus vector of claim 40, wherein said cell-type specific transcriptional regulatory element (TRE) is selected from the group consisting of a prostate-specific TRE (PSA-TRE), a glandular kallikrein-1 TRE (hKLK2-TRE), a probasin TRE (PB-TRE), an α-fetoprotein TRE (AFP TRE) and a carcinoembryonic antigen TRE (CEA TRE).

42. (new) A composition comprising:

a replication competent adenovirus vector for selective cytolysis of a target cell, comprising an E2F-1 transcriptional regulatory element (TRE) operably linked to an adenovirus gene essential for replication selected from the group consisting of E1A, E1B and E4; and

a pharmaceutically acceptable excipient.

43. (new) An isolated host cell comprising the adenovirus vector of Claim 35.

44. (new) A method of propagating adenovirus *in vitro*, the method comprising:

a replication competent adenovirus vector for selective cytolysis of a target cell, comprising an E2F-1 transcriptional regulatory element (TRE) operably linked to an adenovirus gene essential for replication selected from the group consisting of E1A, E1B and E4 wherein said cell is maintained under cell cycling conditions *in vitro*, thereby expressing said adenovirus gene essential for replication;

wherein said adenovirus is propagated.

45. (new) The method of Claim 44, wherein said propagating of said adenovirus is cytotoxic to said cell.

46. (new) The method of Claim 44, wherein said cell is a tumor cell.

Tumor-selective transgene expression *in vivo* mediated by an E2F-responsive adenoviral vector

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Recent data suggest that many tumors, such as malignant gliomas, have disrupted pRB function, either because of *RB-1* gene mutations or as a result of mutations affecting upstream regulators of pRB such as cyclin D1 or p16/INK4a/MTS1 (ref. 1–5). Tumor suppression by pRB has been linked to its ability to repress E2F-responsive promoters such as the EZF-1 promoter[6,7]. Thus, a prediction, which has not yet been demonstrated experimentally *in vivo*, is that E2F-responsive promoters should be more active in tumor cells relative to normal cells because of an excess of "free" E2F and loss of pRB/E2F repressor complexes. We demonstrate that adenoviral vectors that contain transgenes driven by the E2F-1 promoter can mediate tumor-selective gene expression *in vivo*, allowing for eradication of established gliomas with significantly less normal tissue toxicity than seen with standard adenoviral vectors. Our data indicate that de-repression of the E2F-1 promoter occurs in cancer cells *in vivo*, a finding that can be exploited to design viral vectors that mediate tumor-selective gene expression.

E2F-1 is a ubiquitously expressed, growth-regulated gene, which exhibits peak transcriptional activity in S phase[6,8,9]. The E2F-1 promoter contains four E2F binding sites, which act as repressor elements in quiescent cells[7,10–13]. We constructed an adenoviral vector that utilizes the E2F-1 promoter (base pairs –218 to +51; a segment containing four intact E2F consensus sequences) to drive expression of an *Escherichia coli* β-galactosidase gene, placed in the E1 region of an E1/E3-deleted adenoviral vector (Fig. 1), as previously described[14,15]. Transduction of proliferating C6 glioma cells by Ad.E2F1-βgal resulted in high-level expression of β-galactosidase activity in a titer-dependent manner (data not shown). We next examined whether Ad.E2F1-βgal could mediate cell cycle-dependent transgene expression *in vitro*. C6 cells were serum-starved, transduced by either Ad.E2F1-βgal or Ad.CMV-βgal (an identical vector except for the presence of a constitutively active cytomegalovirus early promoter rather than the E2F-1 promoter; Fig. 1) and then reexposed to serum. As demonstrated in Fig. 2, serum starvation resulted in a relatively con-

stant number of cells in S phase (~10%), without significant cytotoxicity, whereas reexposure to serum resulted in an increased S-phase fraction to 25%, 46% and 60% at 16, 20 and 28 hours, respectively. β-Galactosidase activity was approximately 20 times the background level in the Ad.CMV-βgal transduced, serum-starved cells and remained constant at this level following reexposure to serum. In contrast, Ad.E2F1-βgal-transduced, serum-starved cells expressed relatively low levels of β-galactosidase activity. When these cells were reexposed to serum, however, β-galactosidase activity was rapidly induced and reached 8, 14 and 20 times the baseline at 16, 20 and 28 hours, respectively (Fig. 2; *P* < 0.001). These data are consistent with the suggestion that Ad.E2F1-βgal-mediated transgene expression is induced following entry into and/or progression through the cell cycle. The fact that the level of β-galactosidase expression in Ad.E2F1-βgal-transduced cells 24 hours after serum refeeding was similar [at equivalent multiplicities of infection (MOIs)] to that seen in cells transduced by Ad.CMV-βgal, suggests that the induced/de-repressed E2F-1 promoter can mediate high-level transgene expression. Similar results were obtained with a panel of human malignant glioma cell lines, suggesting that the observations made with the C6 cells were not cell-line specific (data not shown).
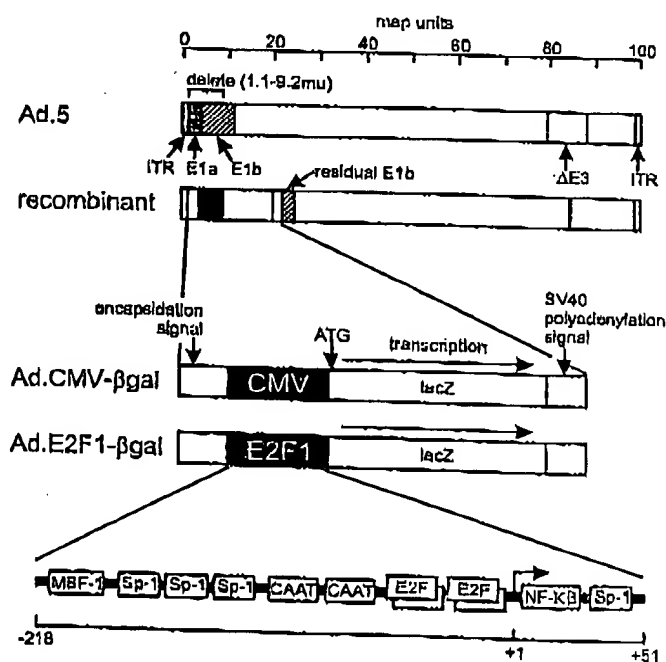

Fig. 1 Adenoviral vectors. The backbone adenoviral sequences are derived from an E3-deleted adenovirus serotype 5, with a bacterial plasmid insert in the E1a region. Following recombination, E1a is replaced by the early CMV promoter (Ad.CMV-βgal) or from the upstream region of the E2F-1 gene (base pairs –218 to +51), a segment containing four intact E2F, 1 NF-κB and four Sp1 consensus sequences. The β-galactosidase gene was derived from a LacZ-containing adenoviral shuttle vector, previously described[15].

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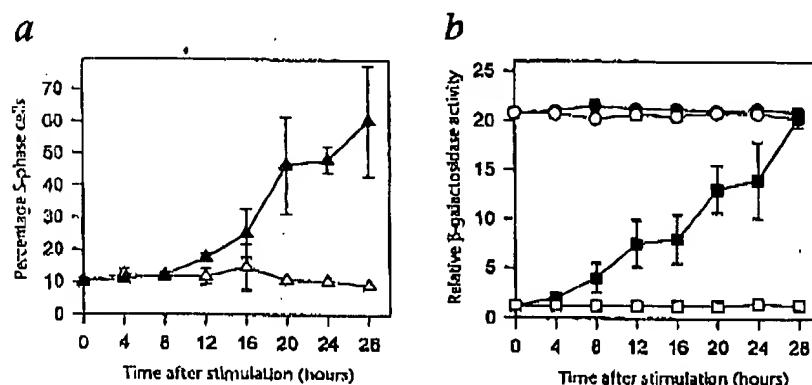
Malignant gliomas represent an excellent model for therapeutic gene transfer, as viral vectors may be injected directly into these relatively localized tumors[16–18]. To test the tumor-selective properties of the Ad.E2F1-βgal vector *in vivo*, we stereotactically injected Ad.E2F1-βgal or the control Ad.CMV-βgal into normal rat brains or brains with established C6 gliomas and killed the animals 3 days later. As can be seen in Fig. 3*a*, injection of Ad.CMV-βgal into normal brain resulted in high levels of β-galactosidase activity in brain adjacent to the needle tract and along ependymal surfaces. Injection of Ad.CMV-βgal into a large, established C6 glioma resulted in widely dispersed β-galactosidase activity predominantly in the tumor and in edematous brain infiltrated by tumor, but also in normal adjacent brain and along ependymal surfaces (Fig. 3*b*) as previously described[19]. In marked contrast, normal rat brains injected with Ad.E2F1-βgal showed virtually no β-galactosidase staining, whereas injection into an established C6 glioma resulted in extensive staining of the tumor itself, with little or no staining in the adjacent normal brain (Fig. 3, *c* and *d*, respectively).

Two possibilities, which are not mutually exclusive, could be invoked to account for the enhanced β-galactosidase activity in tumor tissue relative to normal tissue following injection with Ad.E2F1-βgal. One explanation, given the cell-cycle data described above, is that a higher fraction of glioblastoma cells are cycling than are normal cells found in the surrounding, mitotically quiescent brain. A second explanation is that glioma cells, by virtue of pRB inactivation, contain high-levels of "free" E2F and lack pRB/E2F transcriptional repressor complexes, in which case, even mitotically active normal cells might not achieve the high level of transgene expression observed in tumor cells.

To begin to address these possibilities, we performed two different sets of experiments. In the first set of experiments, rats underwent partial hepatectomy followed 48 hours later by injection of either Ad.CMV-βgal [10⁹ plaque-forming units (PFU)] (Fig. 4, *a*, *c* and *e*) or Ad.E2F1-βgal (10⁹ PFU) (Fig. 4, *b*, *d* and *f*) into the femoral vein. Four days later, livers were harvested and stained for β-galactosidase activity, proliferating nuclear antigen (PCNA) and the adenovirus fiber protein (Fig. 4). Approximately 30% of the hepatocytes were positive for PCNA under these conditions, confirming the presence of a large number of normal proliferating cells in the regenerating livers (Fig. 4, *c* and *d*). Ad.CMV-βgal-transduced livers demonstrated diffuse β-galactosidase staining with no apparent difference between PCNA-positive and PCNA-negative cells (Fig. 4*a*). In contrast, Ad.E2F1-βgal-transduced hepatocytes, including those which were PCNA positive, did not express detectable levels of β-galactosidase (Fig. 4*b*). Detection of adenovirus fiber protein in the liver sections demonstrated similar levels of hepatic transduc-

tion by both the Ad.CMV-βgal and Ad.E2F1-βgal vectors (Fig. 4, *e* and *f*). Similarly, Ad.CMV-βgal, but not Ad.E2F1-βgal, produced high levels of β-galactosidase activity following transduction of nonhepatectomized livers (data not shown). These data suggest that the high-level E2F-1 promoter-mediated transgene expression *in vivo* is not merely a function of active cell cycling.

In the second set of experiments, we constructed a virus identical to Ad.E2F1-βgal, except that mutations were introduced into the four E2F-binding sites within the E2F-1 promoter (Ad.ΔE2F1-βgal). These mutations have previously been shown to render the mutated E2F-1 promoter unresponsive to E2F *in vitro*[10]. In experiments similar to those outlined in Fig. 2, transduction of serum-starved and refed C6 cells *in vitro* by Ad.ΔE2F1-βgal resulted in constitutive transgene expression independent of cell cycle (data not shown). When Ad.ΔE2F1-βgal was stereotactically injected into non-tumor-bearing rat brain, β-galactosidase activity was readily apparent in normal cells (Fig. 3*e*). This is in marked contrast to the lack of β-galactosidase expression in normal cells when the vector contained the intact E2F consensus sequence (Ad.E2F1-βgal; Fig. 3*c*). It is interesting that Ad.ΔE2F1-βgal-mediated β-galactosidase expression in established gliomas *in vivo* was less than that seen with Ad.E2F1-βgal (Fig. 3*f*). These
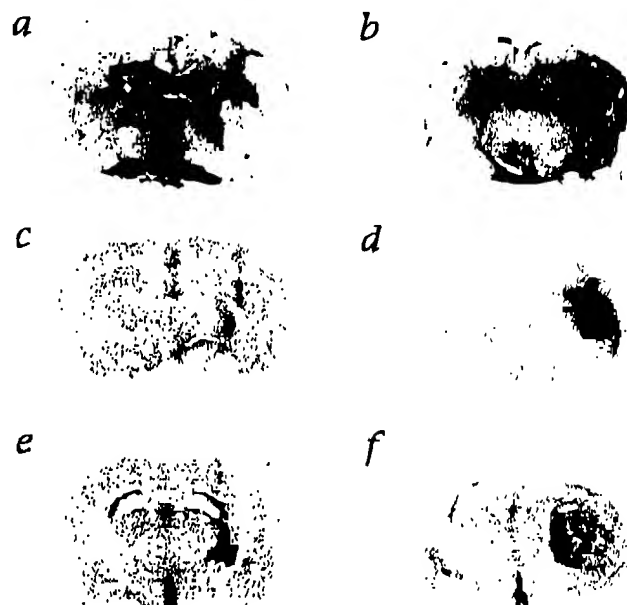
Fig. 3 Ad.E2F1-βgal-mediated tumor selective transgene expression *in vivo*. *a*, Ad.CMV-βgal injected into normal brain; *b*, Ad.CMV-βgal injected into tumor-bearing brain; *c*, Ad.E2F1-βgal injected into normal brain; *d*, Ad.E2F1-βgal injected into tumor-bearing brain; *e*, Ad.ΔE2F1-βgal injected into normal brain; *f*, Ad.ΔE2F1-βgal injected into tumor-bearing brain.
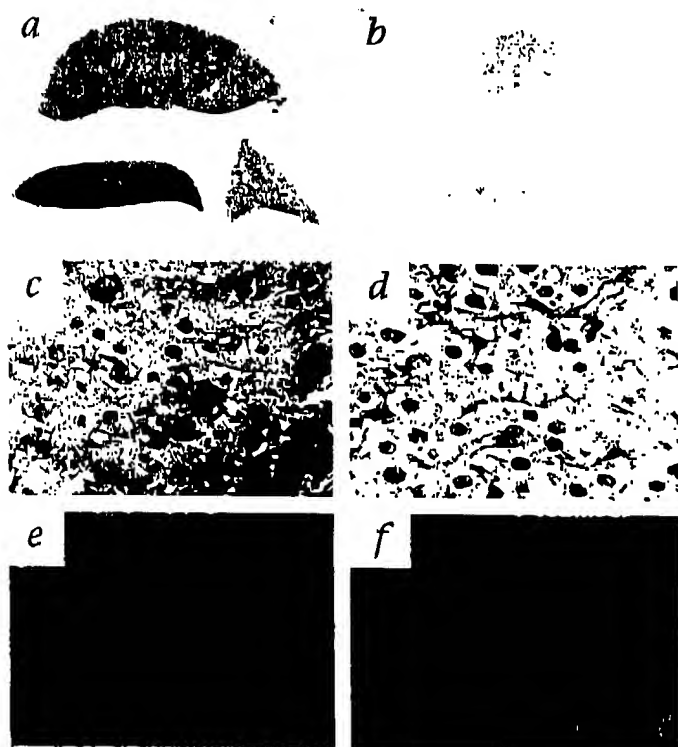
Fig. 4   Ad.E2F1-βgal transduction of normal regenerating liver.   a and b, Whole-mount liver sections stained for β-galactosidase;   c and d, Thin sections of liver tissue stained for β-galactosidase activity and PCNA expression;   e and f, immunofluorescence of thin sections of the liver for expression of the adenoviral fiber protein. a, c and e, Ad.CMV-βgal; b, d and f, Ad.E2F1-βgal.

data suggest that the E2F-responsive sequences within the E2F-1 promoter are essential for the tumor-selective properties of the vector. Furthermore, they strongly suggest that the tumor selectivity of the E2F-responsive vector is due to a combination of factors, namely, repression in normal tissue by pRB/E2F complexes and activation in tumor tissue due to loss of pRB repressor complexes and an excess of free E2F.

To explore whether the tumor-selective properties of this vector could be successfully used for expression of a therapeutic gene, we constructed vectors containing the herpes thymidine kinase (tk) gene driven by either the E2F-1 promoter (Ad.E2F1-tk) or the CMV promoter (Ad.CMV-tk). In vitro transduction of C6 cells by Ad.CMV-tk or Ad.E2F1-tk, demonstrated that both vectors equally sensitized glioma cells to ganciclovir (GCV) [median lethal dose (LD$_{50}$) = 0.05 μM; Fig. 5a]. In order to confirm the activity of the vector in vivo, Ad.CMV-tk or Ad.E2F1-tk were stereotactically injected into 7-day-old intracerebral C6 gliomas, followed by systemic GCV treatment for 1 week (Fig. 5b). There was no significant difference in survival in animals treated with either Ad.CMV-tk or Ad.E2F1-tk plus GCV (>60% of animals without evidence of tumor >3 months from treatment), although animals in both these groups lived significantly longer than animals from the control groups (log rank analysis of Kaplan-Meier survival curves, P < 0.0001; Fig. 5b).

Since the evaluation of toxicity to normal brain tissue in tumor-bearing animals can be difficult as a result of changes induced by necrotic tumor tissue, we elected to evaluate vector-mediated toxicity to normal tissue in the brains of

non-tumor-bearing rats. One week following the injection of either Ad.CMV-tk or Ad.E2F1-tk and systemic GCV therapy, non-tumor-bearing brains were harvested and pathologically evaluated. Consistent with reports in nonhuman primates, stereotactic injection of a tk constitutively expressing adenoviral vector (Ad.CMV-tk) plus GCV resulted in extensive areas of local brain necrosis, inflammation and hemorrhage (Fig. 5c)[20]. In marked contrast, treatment with Ad.E2F1-tk and GCV resulted in no obvious toxicity to normal tissue, except that from the local trauma of the stereotactic injection (indistinguishable from sham-injected animals). These studies confirm that an E2F-responsive adenoviral vector can mediate tumor-selective cytotoxic transgene expression with similar antitumor efficacy, but with significant less toxicity to normal tissue than that of constitutively expressing vectors.

These results suggest that the activity of at least certain E2F-responsive promoters in tumor cells exceeds that achieved in mitotically active normal cells, and this difference can be exploited to target tumor-selective gene expression in vivo. This may be particularly relevant for glioblastomas where the p16-cdk4/cyclin D1-Rb signal pathway is deregulated in the majority of cases[1-3].

The use of highly efficient vectors that incorporate a promoter that can be induced by a deregulated signal transduction pathway commonly found in human gliomas offers a promising method for decreasing the potential for toxicity to normal tissue after intracerebral transduction of cytotoxic genes. Other promising, highly efficient vectors such as those based on the herpes simplex and adenovirus-associated viruses could also exploit E2F-responsive promoters to achieve glioma-selective transgene expression. Additionally, the demonstration that many tumors contain mutations that affect the Rb/E2F pathway suggest that viral vectors incorporating E2F-responsive promoters may ultimately prove useful for gene therapy for a number of diverse malignancies.

Methods

Tumor cell line and animals. The rat C6 astrocytoma cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin. Adult female Sprague-Dawley rats (150–175 g) were purchased from Charles River Laboratories (Wilmington, MA).

Recombinant adenovirus. The recombinant adenoviruses Ad.E2F1-βgal and Ad.CMV-βgal were constructed by homologous recombination between pJM17, a plasmid containing the genome of adenovirus type 5 (Ad5) with deletions in the E1 and E3 regions, and a shuttle plasmid that includes Ad5 sequences (map units 0.0 to 1.3 and 9.2 to 17.3) and the E. coli lacZ gene driven by either the cytomegalovirus (CMV) early gene enhancer/promoter or the E2F-1 promoter. The E2F-1 promoter used in Ad.E2F1-βgal contains the upstream region of the E2F-1 gene (base pairs –218 to +51), a segment containing four intact E2F (two imperfect palindromes) and four Sp1 consensus sequences. Construction of the ΔE2F-1 promoter and the Ad.CMV-tk vector has been previously described[19,21]. The recombinant viruses were plaque purified and propagated in 293 cells as previously described[19]. The structures of the resulting recombinant vectors were confirmed by restriction enzyme digestion and sequencing. The virus stocks were purified by two cesium chloride ultracentrifugations, dialyzed against 10% glycerol, 10 mM Tris (pH 8.0) and 1 mM MgCl$_2$ (viral vehicle) and stored at –80°C. Virus titers were determined as PFU assayed in semisolid cultures of 293 cells.

In vitro studies. Cell-cycle inducibility. C6 cells were serum starved (0.5% FBS) for 48 h and then transduced by Ad.E2F1-βgal, Ad.CMV-βgal or Ad.ΔE2F1-βgal at a multiplicity of infection (MOI) of 100. Serum-starved cells were split into two groups 48 h later, one refed with 10% FBS and the

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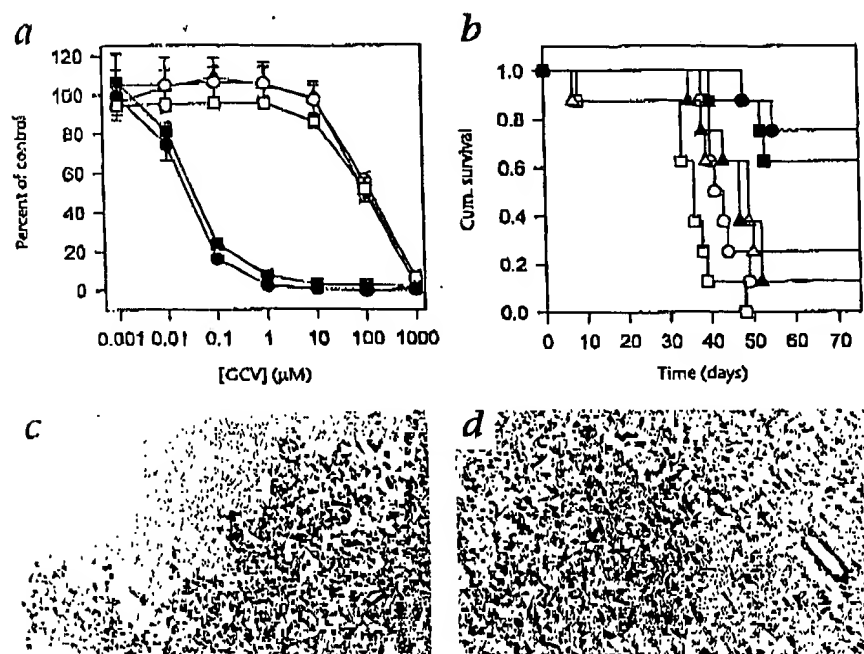
Fig. 5 *In vitro* and *in vivo* antitumor activity of Ad.E2F1-tk. *a*, C6 glioma cells were infected with viral vectors at an MOI of 50 for 1 h, then washed and re-plated in tissue culture plates containing GCV at various concentrations. The cells were fixed 72 h later, stained with methylene blue, and absorbance at 600 nm (A_{600}) was determined on a microplate reader. ($\triangle$) No virus; (O) Ad.CMV-βgal; (●) Ad.CMV-tk; (□) Ad.E2F1-βgal; (■) Ad.E2F1-tk. *b*, Kaplan-Meier survival curves of animals treated by stereotactic injection of viral vectors into 6-day-old established intracerebral C6 gliomas, followed by twice a day treatment with i.p. GCV (15 mg/kg) or saline. (●) Ad.CMV-tk, GCV; (O) Ad.CMV-tk, saline; (■) Ad.E2F1-tk, GCV; (□) Ad.E2F1-tk, saline; (▲) Ad.ΔE2F1-βgal, GCV; (△) Ad.E2F1-βgal, saline. *c* and *d*, Hematoxylin and eosin-stained thin section of normal brain from animals intracerebrally injected with Ad.CMV-tk (*c*) or Ad.E2F1-tk (*d*) and treated with GCV (15 mg/kg) twice daily for 7 days.

other maintained in low serum. Cells were harvested at the indicated time points, fixed with ethanol, stained with propidium iodide or with an anti-β-galactosidase antibody (Boehringer-Mannheim) and analyzed by ELISA.

Cytotoxicity assays. C6 glioma cells were seeded in 75-cm² tissue culture plates, washed and infected with viral vectors at various MOIs for 1 h in 0.5% medium. Cells were then washed and cultured in normal medium for an additional 12 h followed by replating at a density of 500 cells per well in 96-well tissue culture plates containing GCV (Cytovene, Hoffmann-La Roche, Nutley, NJ) at various concentrations. The cells were fixed 72 h later with the addition of formaldehyde (3.7% final), washed extensively with water and stained with 200 µl of 0.05% methylene blue for 15 min. Unbound dye was washed away, and bound dye was released for quantification by addition of 200 µl 0.33 M HCl for 15 min. Absorbance at 600 nm (A_{600}) was determined on a microplate reader after mixing for 5 s.

Implantation of intracerebral tumors. C6 glioma cells were stereotactically implanted into the right caudate nucleus of Sprague-Dawley rats using a modification of the method of Kobayashi[24]. In brief, rats (150–175 g) were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg) and placed in a small animal stereotaxic frame (Kopf Instruments, Tujunga, CA). A sagittal incision was made through the scalp to expose the skull, and a small burr hole was made 1.3 mm posterior and 4 mm to the right of the bregma. Twenty thousand tumor cells, suspended in 10 µl of HBSS, was injected with a 701 Hamilton syringe over 30 s to a depth of 4.5 mm. The needle was left in place for 1 min and then withdrawn slowly. The hole in the skull was plugged with bone wax, and the incision was closed with surgical clips (Ethicon Plus, Franklin Lakes, NJ). This method resulted in a 100% yield of intracerebral tumors with relatively little leptomeningeal or intraventricular spread.

Vector injections. Ad.E2F1-βgal (10⁹ PFU), Ad.CMV-βgal (10⁹ PFU) or Ad.ΔE2F1-βgal (10⁹ PFU) was stereotactically injected into normal brain or 14-day-old intracerebral C6 gliomas utilizing the same coordinates and methods described above. Animals were killed 3 days later, and brains were harvested for further studies.

For the thymidine kinase studies, Ad.CMV-tk (5 × 10⁸ PFU), Ad.E2F1-tk (5 × 10⁸ PFU), or Ad.E2F1-βgal (5 × 10⁸ PFU) was injected into 6-day-old established gliomas followed by i.p. injections of GCV (15 mg/kg) or saline twice a day for 7 days.

Hepatectomy and intrahepatic vector injections. Sprague-Dawley rats

(150–175 g) were anesthetized with an i.p. injection of pentobarbital (40 mg/kg). The hepatic artery, portal vein and bile ducts leading to the middle and left lobes of the liver were identified and ligated, allowing removal of these two lobes (70% hepatectomy). Forty-eight hours after hepatectomy or sham operation (controls), Ad.E2F1-βgal (10⁹ PFU) or Ad.CMV-βgal (10⁹) was injected into the right femoral vein. Animals were killed 4 days later, and livers were harvested for further studies.

Tissue preparation. Animals were killed by pentobarbital overdose followed by cardiac perfusion, first with ice-cold PBS, followed by 4% paraformaldehyde/PBS. Brain or liver tissue was harvested whole and further fixed in 4% paraformaldehyde/PBS for a further 2 h at 4 °C and then sequentially washed over 3 nights in 10%, 20% and 30% sucrose in PBS/2 mM MgCl. Brain was then cut coronally along the needle track, and liver lobes were cut lengthwise and slices used directly for whole mounts or embedded in OCT compound (Tissue Tek, Elkhart, IN) and frozen for thin sections.

For X-gal staining of whole mounts, the fixed samples were rinsed once with cold PBS and incubated in PBS solution containing 2 mM CaCl₂, 0.01% sodium deoxycholate and 0.02% NP-40 for 10 min at 4 °C. Samples were then stained for 6 h at 37 °C in the same solution containing 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆ and 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), mounted and photographed.

For thin sections, 6-µm sections were cut using an IEC Minotome cryostat (IEC, Needham, MA) and placed on poly-lysine-coated slides. Slides were allowed to air-dry and stored at −20 °C. For staining of liver sections, slides were briefly washed in PBS, fixed with cold acetone and allowed to air-dry. X-gal staining was then carried out overnight (18 h) with the X-gal staining solution as described above. Anti-PCNA primary antibody (PC10; Dako, Carpinteria, CA) or polyclonal anti-adenovirus anti-serum was diluted 1:200 in PBS/BSA/Tween-20 and applied for 30 min followed by vigorous washing (Chemicon Int. Inc, Temecula, CA). A biotinylated horse anti-mouse IgG secondary antibody was used with a peroxidase ABC system (Vectastain, Vector Laboratories, Burlingame, CA) and DAB with nickel enhancement as the substrate, as previously described. Stained sections were counterstained with nuclear fast red (Vector Laboratories) to highlight cell nuclei. The anti-adenovirus monoclonal antibody was detected using a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Chemicon Int. Inc.) and visualized under fluorescent microscopy. Standard hematoxylin and eosin staining of brain sections was also carried out after the slides were fixed in cold methanol.

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